Supplementary Information

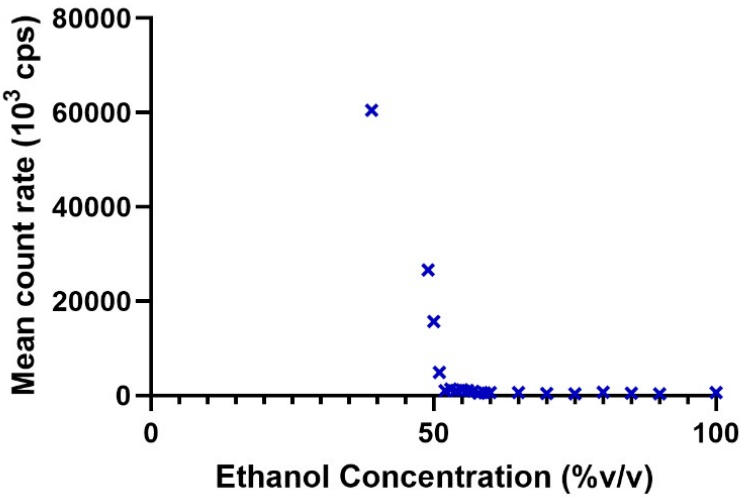
Formation of RNA Lipid Nanoparticles: An Equilibrium Process with a Liquid Intermediate Stage

Jessica Boutros, Ziyue Li, Leah Wright, Robert J. Falconer

School of Chemical Engineering, University of Adelaide, North Terrace, Adelaide, SA 5005, Australia.

Solubility of the lipid mixture of SM102, DSPC, cholesterol, and DMG-2000 in water-ethanol mixtures

Titration of 10 mM sodium acetate buffer (pH 4) into SM102, DSPC, cholesterol, and DMG-2000 in ethanol (Supplementary Figure 1), confirmed that the mixture was soluble in 52 %(v/v) ethanol. Dynamic light scattering did not detect any particle formation at 52 %v/v ethanol.



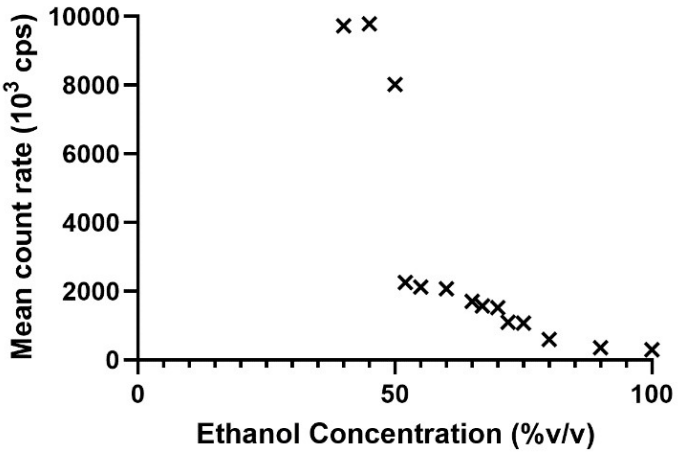
Supplementary Figure 1: Measurement of the count rate at different ethanol concentrations using dynamic light scattering. 10 mM sodium acetate buffer, pH 4 was titrated into 1000 µl SM102, DSPC, cholesterol, and DMG-2000 at molar ratio 50:38.5:10:1.5 at 1 mg/ml. equilibration time is set around 2 mins.

Isothermal titration calorimetry of SM102, DSPC, cholesterol, and DMG-2000 titrated into tRNA

The intensity of the power signal in the isothermal titration calorimetry thermogram was too great for detailed analysis. The signal did return to the baseline before the next injection which demonstrated that equilibrium was achieved before the next injection.

Solubility of SM-102 in water-ethanol mixtures

SM-102 lipid stocks were prepared in ethanol. Sodium acetate buffer pH 4 was titrated gradually into 400 µL of lipid in ethanol. The equilibration time is set for 120 seconds to allow the formation of the LNP to produce. Dynamic light scattering (DLS) was used to detect the ethanol concentration where particles started to form (Supplementary Figure 2).

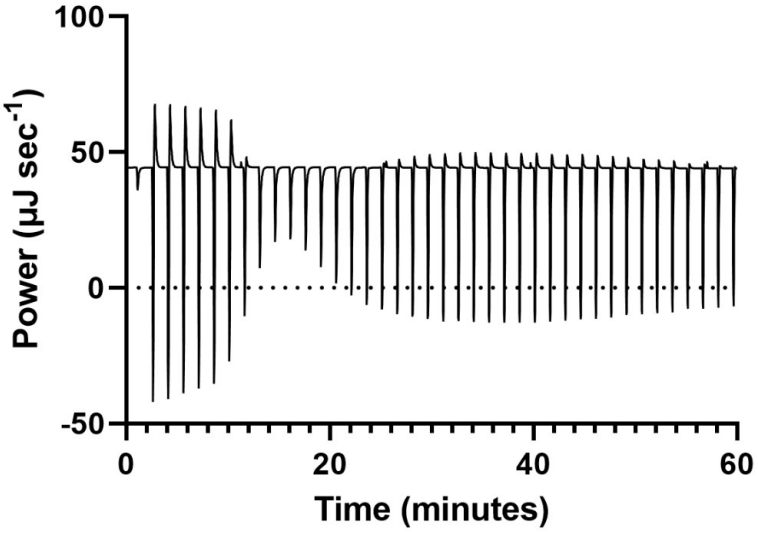


Supplementary Figure 2: Measurement of the count rate at different ethanol concentrations using dynamic light scattering. 10 mM sodium acetate buffer, pH 4 was titrated into 1000 µl 2 mM SM102. Equilibration time is set around 2 mins.

Titration of 10 mM sodium acetate buffer (pH 4) into SM-102 in ethanol (Supplementary Figure 2), suggested that SM-102 is soluble in 52 %(v/v) ethanol. Dynamic light scattering detected a major transition around 50 %(v/v) ethanol. The low count rate at ethanol concentrations between 50 and 80 %v/v suggests that there is self-association between SM-102 molecules at higher ethanol concentrations. The DLS estimated the hydrodynamic diameter of SM-102 clusters in high ethanol (>50 %v/v)) as being between 200-400 nm but there was a high PDI and no trend between size and ethanol concentration.

Isothermal titration calorimetry of SM-102 titrated into tRNA

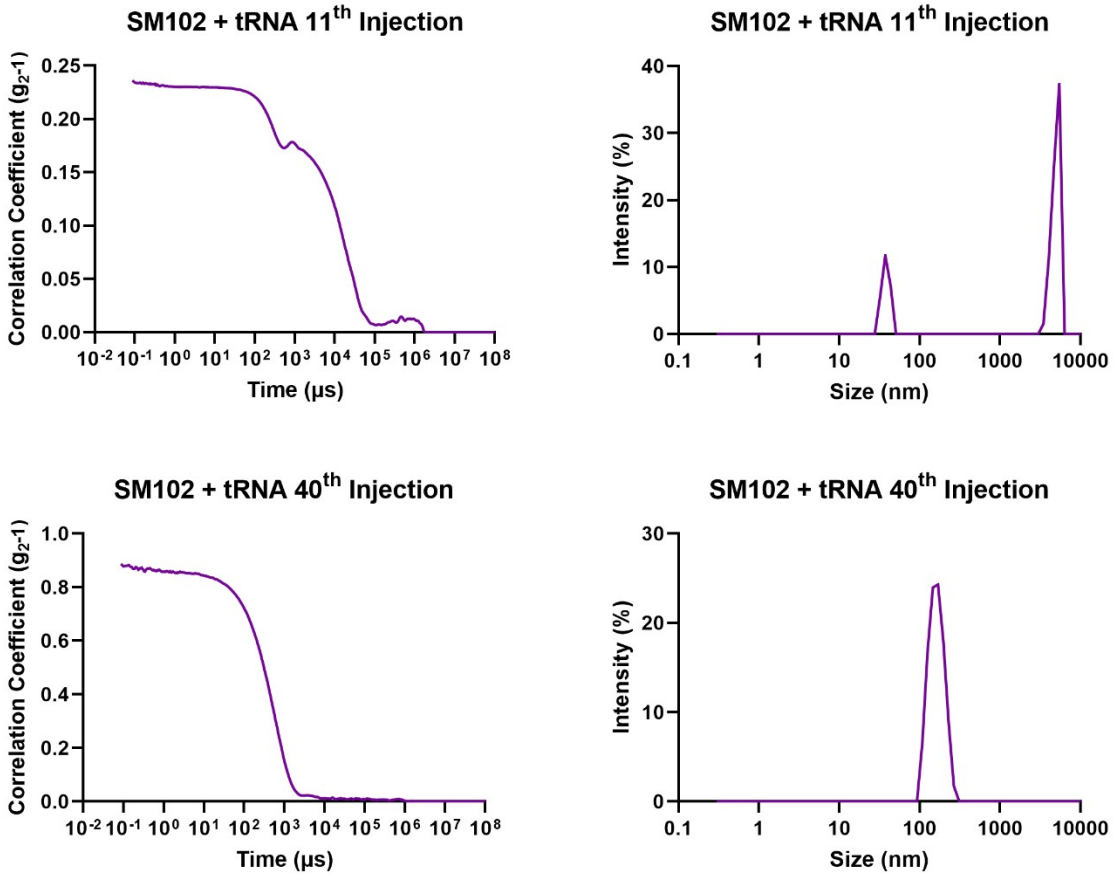
The thermogram for SM-102 titration into tRNA demonstrates that equilibrium is achieved before the next injection and that the experiment is comprised of a series of 40 individual equilibrium reactions. The thermogram is complex and would require a series of experiments to understand the role of electrostatic, possible hydrogen bonding and hydrophobic lipid-lipid interactions in LNP assembly. This is outside the scope of this paper.



Supplementary Figure 3. Isothermal titration calorimetry thermogram for 2mM SM-102 in 52 %(v/v) ethanol and 48 %(v/v) 10 mM acetate buffer, pH 4.0; titrated into 0. 1096 mM tRNA in 40 %(v/v) ethanol and 60 %(v/v) 10 mM acetate buffer, pH 4.0 at 25 °C.

Dynamic light scattering analysis of samples taken from SM-102 titrated into tRNA

The graph showing correction coefficient versus decay time for SM-102 injection into tRNA at the 11th injection (Supplementary Figure 4), is typical for a sample with a mixture of particle sizes and is consistent with the high polydispersity index (PDI) of 0.76 +/- 0.13. Conversely, the graph showing correction coefficient versus decay time for SM-102 injection into tRNA at the 40th injection is typical for a sample with a small range of particle sizes and is consistent with the relatively low polydispersity index (PDI) of 0.16 +/- 0.09.



Supplementary Figure 4: Dynamic light scattering analysis of 2mM SM-102 in 52 %(v/v) ethanol and 48%(v/v) 10 mM acetate buffer, pH 4.0; titrated into 40% (v/v) ethanol and 60% (v/v) 10 mM acetate buffer, pH 4.0 at 25 °C; showing the correction coefficient versus decay time and the size distribution. The 11th injection corresponds to a N/P ratio of 0.95:1 and the 40th injection to 3.6:1.

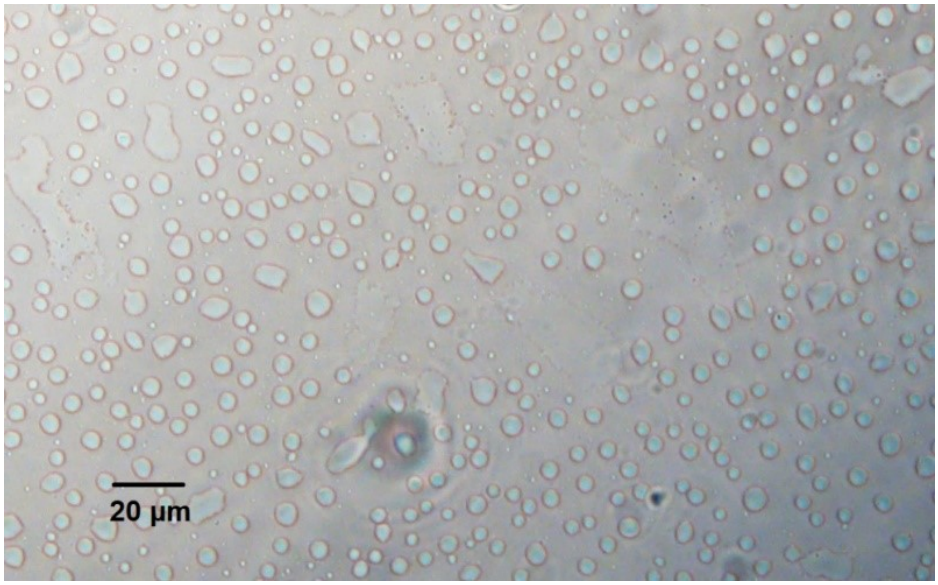
Further evidence for coacervates between RNA and different cationic lipids and lipid mixtures

Titration of 2mM SM-102, 1.54 mM cholesterol, 0.4 mM helper lipid DSPC, 0.06 mM DMG-PEG2000 in 52 %(v/v) ethanol into 0.05 mM tRNA in 40 %(v/v) ethanol (pH 4.0) is a similar experiment in the main manuscript, except the ethanol concentration in the solution containing the tRNA was 40 %v/v ethanol (this ethanol concentration is the same as the SM-102 and ALC-0315 titration experiments (Figures 3, 4 and 5, and Supplementary Figure 6).

Method

In the experiment on the equilibrium process for Spikevax lipid formulation tRNA lipid nanoparticles, the syringe contained 2mM SM-102 in 52 %(v/v) ethanol and 48 %(v/v) 10 mM acetate buffer, pH 4.0. The cell contained 0.04647 mM tRNA in 40 %(v/v) ethanol and 60 %(v/v) 10 mM acetate buffer,

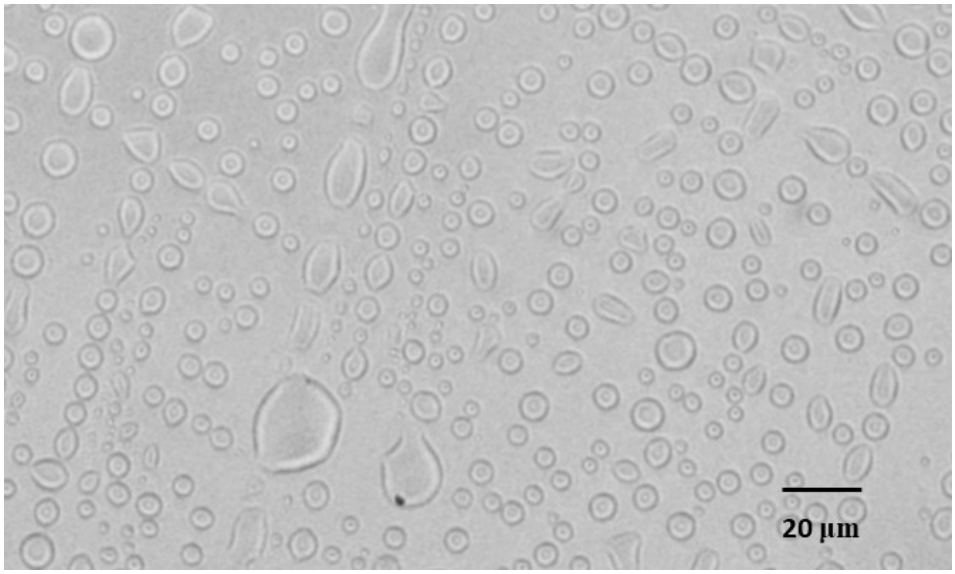
pH 4.0. Each injection lasted for 2 seconds, utilizing the high reference feedback system. The reference power was maintained at 41.8 µW.



Supplementary Figure 5. Light microscopy of 2mM SM-102, 1.54 mM cholesterol, 0.4 mM helper lipid DSPC, 0.06 mM DMG-PEG2000 in 52 %(v/v) ethanol and 48 %(v/v) 10 mM acetate buffer, pH 4.0; titrated into 0.05 mM tRNA in 40 %(v/v) ethanol and 60 %(v/v) 10 mM acetate buffer, pH 4.0 at the 11^{th} injection where the N/P ratio of 1:1.

Method

In the experiment on the equilibrium process for ALC-0315 tRNA lipid nanoparticles, the syringe contained 2mM SM-102 in 52 %(v/v) ethanol and 48 %(v/v) 10 mM acetate buffer, pH 4.0. The cell contained 0.04647 mM tRNA in 40 %(v/v) ethanol and 60 %(v/v) 10 mM acetate buffer, pH 4.0. Each injection lasted for 2 seconds, utilizing the high reference feedback system. The reference power was maintained at 41.8 µW. Each ITC experiment was undertaken in triplicate.



Supplementary Figure 6. Light microscopy of 2mM ALC-0315 in 52 %(v/v) ethanol and 48 %(v/v) 10 mM acetate buffer, pH 4.0; titrated into 0.1096 mM tRNA in 40 %(v/v) ethanol and 60 %(v/v) 10 mM acetate buffer, pH 4.0 at the 11th injection where the N/P ratio of 0.95:1. Note, evidence of coalescence on the microscopy slide consistent with the two liquid phases.

DLS was also conducted on the 11th and 40th injection of ALC-0315 into tRNA. It also showed a reduction from larger coacervate droplets to smaller LNPs as observed with SM-102.

Supplementary Table 1: DLS results for lipid mixture containing 2 mM ALC-0315 in 52% ethanol, 48% 10 mM sodium acetate buffer pH 4, titrated into 0.1 mM tRNA in 40% ethanol, 80% 10 mM sodium acetate, pH 4 buffer using an ITC.

	11th injection	40th injection
N/P ratio	0.95:1	3.6:1
Mean hydrodynamic diameter	480 ± 120 nm	134 ± 3 nm
Polydispersity index (PDI)	0.55 ± 0.14	0.16 ± 0.14